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COMPARATIVE BIOCHEMISTRY AND METABOLISM PART II: NAPHTHALENE LUNG TOXICITY

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This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

Roger C. Inman

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Earlier studies have shown that cytochrome P-450 dependent metabolism of the volatile aromatic hydrocarbon, naphthalene, results in dose dependent bronchiolar necrosis in mice and in the formation of reactive metabolites which deplete cellular glutathione and become bound covalently to tissue macromolecules. Pretreatments which altered the severity of bronchiolar necrosis altered the extent of covalent binding in the lung. However, the overall levels of covalently bound metabolites in liver and kidney (non target tissues) were

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higher than in lung suggesting that several different reactive metabolites might be formed in the tissue but not all are toxicologically important or that the liver may contribute substantially to the overall levels of covalent binding in extrahepatic organs. To examine potential differences in target vs. non target organ metabolism of naphthalene to chemically reactive metabolites in vitro a high pressure liquid chromatographic method has been developed to separate and quantitate glutathione adducts formed from reactive naphthalene metabolites generated in microsomal or tissue slice incubations. At least three such metabolites were found in microsomes or tissue slices; these have not yet been identified. In addition, the quantitative formation of these adducts in tissue slices from target vs. non target tissue differed substantially. Considerable evidence also indicated that reactive metabolites of naphthalene formed in the liver may efflux from this organ and become bound covalently to macromolecules in lung and kidney. This view is supported by the following: A) kidney microsomes formed covalently bound metabolites from naphthalene at very low rates yet in vivo covalent binding was very high in the kidney, B) pretreating mice with phenobarbital resulted in a 2 fold increase in covalent binding in lung after 400 mg/kg ^{14}C-naphthalene in vivo but a decrease in lung microsomal formation of reactive metabolites in vitro, C) pretreatment with p-xylene, a selective inhibitor of pulmonary cytochrome P-450 decreased covalent binding and glutathione depletion by 300 mg/kg ^{14}C-naphthalene only slightly, D) pretreatment with a selective depletor of glutathione in liver and kidney (buthionine sulfoximine) markedly increased covalent binding in lung as well as liver and kidney, and E) administration of naphthalene by inhalation resulted in only a slight decrease in pulmonary glutathione levels. Continuing studies are aimed at confirming the differences in target vs non target organ metabolism of naphthalene and studying metabolism in lungs of sensitive vs non sensitive species with the intention of extending the studies to human lung tissue. Studies are also continuing to examine the question of binding of reactive metabolites of naphthalene to DNA.

PREFACE

This is the annual report of the Subprogram on Comparative Biochemistry and Metabolism, Part II: Naphthalene Lung Toxicity and concerns work performed by the Department of Community and Environmental Medicine of the University of California, Irvine, on behalf of the Air Force under Contract #F33615-80-C-0512, Work Unit 63020115. This document describes the accomplishments of the subprogram from June 1981 through May 1982.

A.R. Buckpitt served as coordinator of Part II of the subprogram. Acknowledgment is made to Dr. Ronald Shank for his advice and encouragement in the DNA binding studies, to William Bosan for his help in the GC/MS identification of the glutathione conjugates, to Eric Hunt for his advice and help in the analysis of binding to DNA, and to Paul Williamson, Greg Smart and Darren Warren for their contributions to the research program.

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INTRODUCTION

This report summarizes the work conducted from June 1981 through May 1982 in the subprogram on Comparative Biochemistry and Metabolism, Part II: Naphthalene Lung Damage. The mission of this subprogram is to provide further understanding of the relevant biochemical/metabolic events associated with the selective pulmonary bronchiolar epithelial cell necrosis observed in naphthalene treated mice. The rationale for these studies and the importance to the USAF/USN will be discussed briefly in the following paragraphs.

Naphthalene is an ubiquitous environmental contaminant which is found in cigarette smoke and as a contaminant in air and water. Yearly, more than 300,000 metric tons of naphthalene are used in industry, primarily as a feed stock in the synthesis of dye intermediates (Priority Toxic Pollutants, 1980). Naphthalene is a starting material in the synthesis of decalin and tetrahydronaphthalene and is present in relatively large amounts in a solvent for ferrocene, a fuel additive currently under consideration for use by the USAF/USN (MacEwen and Vernot, 1981). Administration of naphthalene either intraperitoneally or as a vapor results in severe bronchiolar epithelial cell necrosis in mice, which appears to depend upon metabolism of the parent compound by the cytochrome P-450 monooxygenases (Warren et al., 1982). Since the bioactivation of chemically inert environmental agents may play a role in lung diseases such as fibrosis, emphysema and cancer (Boyd, 1980; Brody et al., 1981) it is important to understand factors which might influence the balance between toxifying and detoxifying pathways.

In addition, the elucidation of the toxicologically important biochemical and metabolic events occurring in naphthalene exposed animals combined with the development of appropriate in vitro techniques for studying these processes will allow the determination of the potential sensitivity of human lung tissue to aromatic hydrocarbons like naphthalene. The mouse is substantially more sensitive to bronchiolar or alveolar cell damage induced by carbon tetrachloride (Boyd et al., 1980), 3-methylfuran (Boyd et al., 1978), butylated hydroxytoluene (Adamson et al., 1977), bromobenzene (Reid et al., 1973), and naphthalene (Buckpitt, 1981) than is the rat. Yet sufficiently detailed studies on the mechanism for the lung damage by these agents which would allow the appropriate studies to be conducted in human lung tissue have not been reported. Indeed, with few exceptions, studies on the metabolism of chemicals by human lung tissue have focused on benzo(a)pyrene (Prough et al. 1977; Sipal et al., 1979; McManus et al. 1980; Oesch et al., 1980). The ability of human lung tissue to metabolize those chemicals known to damage rodent lungs via the formation of toxic metabolites has not been explored. Since the mouse is used extensively in both short and long

term toxicity testing, it is important to determine whether this species is an appropriate animal model for the human.

BACKGROUND

The pulmonary bronchiolar necrosis resulting from intraperitoneal administration of naphthalene was first reported by Reid et al. (1973). Subsequent light and electron microscopic examination of the lungs of naphthalene treated mice revealed that the non-ciliated bronchiolar epithelial cell (Clara cell) was the primary target cell for naphthalene-induced damage and that the lung lesion was both time and dose-dependent (Mahvi et al., 1977). The finding that the Clara cell is a major locus of pulmonary cytochrome P-450 monooxygenases (Philpot and Wolf, 1981) and that naphthalene is metabolized to a number of reactive and potentially toxic epoxides and diol epoxide derivatives (Horning et al., 1980; Stillwell et al., 1981) suggested that naphthalene-induced bronchiolar damage may not be due to the parent compound but to the cytochrome P-450 dependent formation of toxic metabolites. Our initial studies which have been described in detail (Shank et al., 1980; Buckpitt, 1981; Warren et al., 1982) indicate that metabolism of naphthalene by the cytochrome P-450 monooxygenase results in the formation of highly reactive metabolites which bind covalently to tissue macromolecules in vivo and which appear to conjugate with glutathione. Significant covalent binding and bronchiolar damage does not occur until doses of naphthalene are administered that are sufficient to deplete tissue glutathione substantially. Pretreatment with piperonyl butoxide blocks naphthalene-induced bronchiolar damage and glutathione depletion and covalent binding of reactive metabolites. Likewise, prior depletion of tissue glutathione by diethyl maleate markedly increases covalent binding and pulmonary damage. While these studies supported a relationship between the formation and covalent binding of reactive metabolites and the bronchiolar damage observed after naphthalene, the pattern of organ toxicity did not coincide with the target organ for covalent binding of reactive metabolites (that is, high levels of binding occurred in lung, liver and kidney, but tissue damage was observed only in lung).

There are several possible explanations for this which are still consistent with a role of reactive metabolite formation and tissue damage. Metabolism of naphthalene may result in the formation of several different reactive species (1,2-oxide, 1,2,3,4-diepoxide or diol epoxide metabolites) all of which may be capable of binding irreversibly to tissue macromolecules but which may differ in their ability to interact with macromolecules critical to the survival of the cell. Thus, the nature of reactive metabolites produced could determine the organ specificity for damage. Other possibilities are that the macromolecules to which reactive naphthalene metabolites

bind differ in target and non target tissue or that binding is highly localized in the lung but not in the liver or kidney. Additionally, the results of our early studies indicated that the liver may form reactive naphthalene metabolites which reach the lung via the circulation and which may or may not be toxicologically important. Much of the emphasis of the studies conducted during the past year has been to develop appropriate methodology for studying the formation of reactive naphthalene metabolites in vitro and to attempt to discern the role of the liver in the formation and export of naphthalene metabolites which become bound covalently in extrahepatic tissue.

RESEARCH PROGRAM

NATURE AND RATE OF GLUTATHIONE ADDUCT FORMATION IN VITRO

Electrophilic metabolites produced from the P-450 monooxygenase mediated metabolism of a number of xenobiotics are normally too reactive to be isolated from biologic sources but can often be trapped as thiol adducts with the nucleophile, glutathione (Chasseaud, 1979). Studies on the structure and rates of formation of such metabolites not only can yield valuable information on the nature of the reactive metabolite formed but can also be useful for examining differences in target and non-target tissue formation of a particular reactive metabolite. Since previous in vivo studies had demonstrated that glutathione plays an important role in modulating pulmonary damage and the covalent binding of reactive naphthalene metabolites, studies on the in vitro formation of naphthalene glutathione adducts were thought to be likely to discern differences in target and non-target tissues in the rates or kinds of reactive metabolite(s) formed. Although mercapturic acid and glutathione conjugates of naphthalene have been reported previously (Jeffrey and Jerina, 1975), conjugation was thought to occur solely at the C-2 position. Recent studies with both aromatic (benzo(a)pyrene-4,5-oxide, Armstrong et al., 1981) and aliphatic (styrene oxide, Brown et al., 1982) epoxides have shown that nucleophilic attack can occur at both electrophilic carbon atoms, thus suggesting that the isolation methods utilized in the earlier studies with naphthalene could not adequately separate the various conjugate isomers.

Development of HPLC Methods

The studies conducted during the past contract year have focused on developing an appropriate HPLC method for separating and quantitating the various possible naphthalene glutathione adducts. The methods were developed by comparing the UV and radiochromatographic profiles obtained from microsomal incubations done in the presence or absence of glutathione.

Liver microsomes were prepared from phenobarbital pretreated (0.1% in the drinking water for 6 days, replaced with fresh water 24 hr before sacrifice), male Swiss Webster mice (Charles River Breeding Labs, Wilmington, MA) by differential ultracentrifugation as described by Buckpitt and Boyd (1980). Supernatant enzymes, containing the glutathione S-transferases, were removed from the first 100,000 x g centrifugation and were chromatographed on a 19 x 5 cm Sephadex G-25 column in 0.05 M sodium phosphate buffer pH 7.4 to remove endogenous glutathione. Cytosolic enzymes eluting in the column void volume were collected and used in the incubations.

Incubations were prepared on ice in a total volume of 2 ml and consisted of: 6 mg microsomal protein, ^{14}C-naphthalene (0.5 mM, approximately 1000 dpm/nmole), 2 mg cytosolic enzymes and NADPH generating system (NADP (1 µmole) glucose-6-phosphate (30 µmoles), and glucose-6-phosphate dehydrogenase (1 i.u.)), and were done in sealed incubation vials for 30 min at 37°C in the presence or absence of 5 mM glutathione. Incubations were terminated by the addition of 2 ml ice cold methanol, and the precipitated protein was removed by centrifugation. Attempts to chromatograph aliquots of the methanol/H_2O supernatant from incubations done in the presence of glutathione with a number of different column/solvent system combinations resulted in the elution of a very broad UV and radioactive peak (retention 5-15 min depending upon column and mobile phase) which was not present in extracts from incubations with glutathione. This broad peak was subsequently resolved into 3 distinct peaks when the methanol/water supernatant was evaporated to dryness, reconstituted in mobile phase and chromatographed on a C_{18} µ Bondapak column (0.78 x 30 cm) in 15% methanol/1% glacial acetic acid/84% water at 3 ml/min. UV and radioactive profiles of extracts prepared from identical incubations done in the presence (Figure 1A) or absence (Figure 1B) of glutathione indicate that the peaks eluting at 20, 21 and 24 min are likely to be glutathione adducts; the peak eluting at 27 min was present in extracts prepared from incubations done either in the presence or absence of glutathione.

UV and radioactive peaks were observed at 20, 21 and 24 min in extracts prepared from incubation of microsomes, NADPH generating system, naphthalene (0.5 mM) and ^{3}H-glutathione (L-glycine-2-^{3}H, New England Nuclear 0.1 mM, 2000 dpm/nmole) which were not present in extracts from identical incubations done in the absence of NADPH generating system (data not shown).

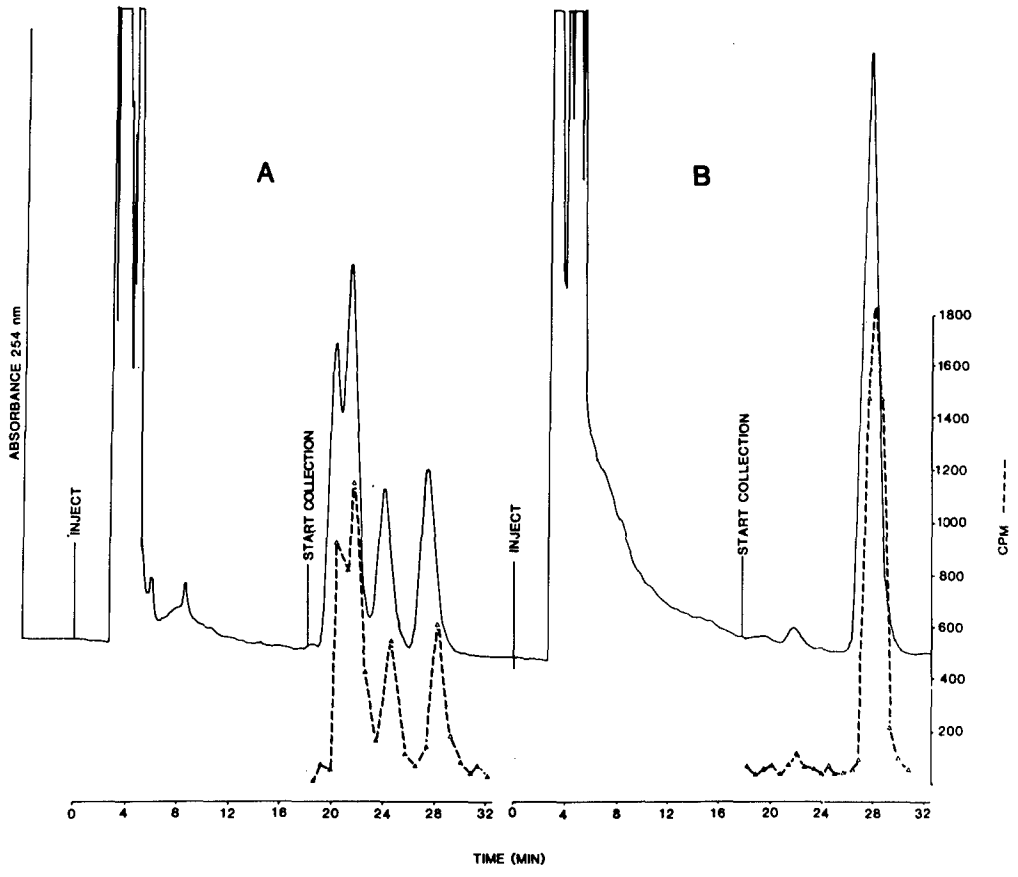


Figure 1. UV and radioactive elution profiles of an extract prepared from an incubation of phenobarbital induced liver microsomes, ^{14}C-naphthalene, NADPH regenerating system and A) reduced glutathione or B) in the absence of glutathione. Aqueous extracts were injected onto a C_{18} μ Bondapak column (0.78 x 30 cm) with a mobile phase of 15% methanol 1% glacial acetic acid/84% water at a flow rate of 3 ml/min. Absorbance was monitored at 254 nm (0.01 AUFS). Thirty second fractions of the column eluate were collected directly into liquid scintillation vials and were counted for 20 min each.

Covalent Binding and Formation of Naphthalene Glutathione Adducts in Lung and Liver Slice Incubations

Studies on the time-course formation of covalently bound and water soluble glutathione adducts from naphthalene in lung and liver slices have been started. Male Swiss Webster mice (20-30 g) were sacrificed by cervical dislocation and lungs were perfused with 3 ml heparinized saline.

Perfused lungs (150-330 mg) were weighed, finely chopped with a razor blade and incubated in 3 ml 25 mM HEPES buffer, pH 7.4 with 0.5 mM ^{14}C-naphthalene (1000 dpm/nmole) for times ranging from 0-60 min. Sections of liver (110-440 mg) were treated in an identical fashion. At the specified incubation time, the contents of each flask were transferred to an ice bath and were frozen at -80°C. The contents of each flask were thawed and homogenized; a 2 ml aliquot was transferred to a centrifuge tube containing 2 ml ice cold methanol. After thorough mixing, precipitated protein was removed by centrifugation. The entire methanol/water supernatant was filtered through a 0.5 μ type FH millipore filter into a clean centrifuge tube. The methanol/water supernatant was extracted 2 times with 4 ml trimethylpentane to remove unchanged naphthalene and was then evaporated to dryness under vacuum. The residue was redissolved in 500 μl water for HPLC analysis.

Figure 2 compares the radiochromatographic profile of an extract prepared from a 60 min lung or liver slice incubation. Lung slices produced considerably more of the conjugates eluting at 21 and 22 min than did liver slices. The putative conjugate eluting at 25 min was found in approximately equal amounts in lung and liver slice incubations. In contrast, the radioactive peak eluting at 28 min, which did not depend upon the presence of glutathione in the incubations, was present in considerably greater amounts in extracts of lung versus liver slices. Although these results must be considered preliminary, they do indicate that there may be differences in lung versus liver metabolism of naphthalene.

Identification of Conjugates

Approximately 100 nmoles of each of the naphthalene glutathione conjugates were collected, and the solvent was removed by rotary evaporation. Each compound was shown to be chromatographically homogeneous by reinjecting a small quantity of each sample onto a C_{18} μ Bondapak column (0.78 x 30 cm) run in 15% methanol/1% acetic acid/84% water at 3 ml/min.

The initial strategy for identifying these conjugates has been similar to that used recently by Armstrong et al. (1981) for identifying the positional glutathione conjugate isomers of

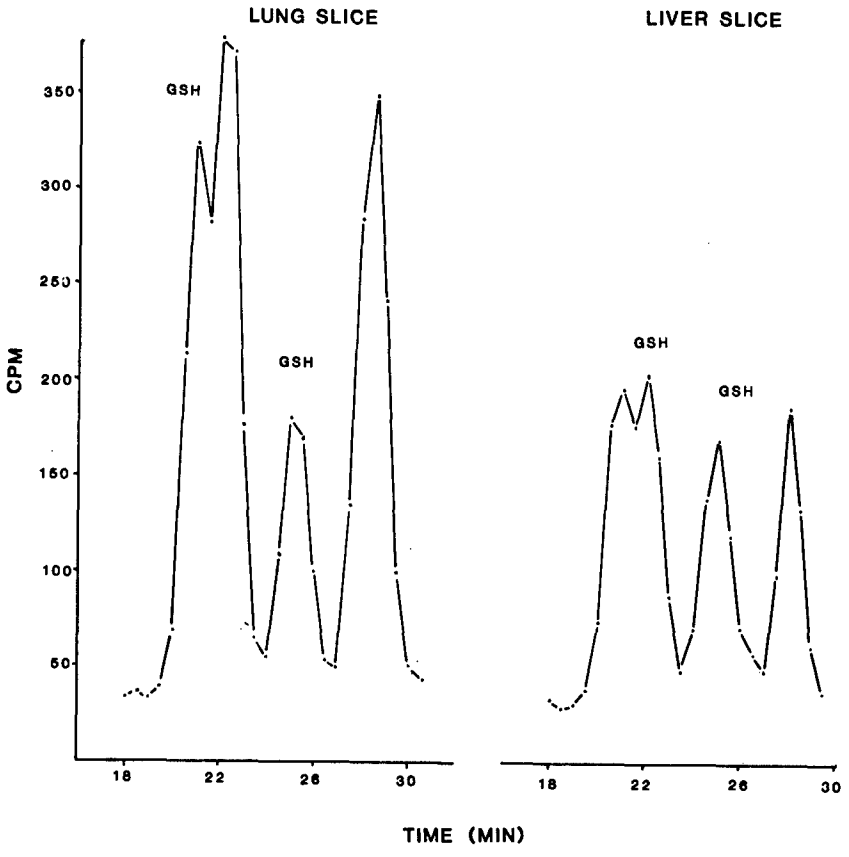
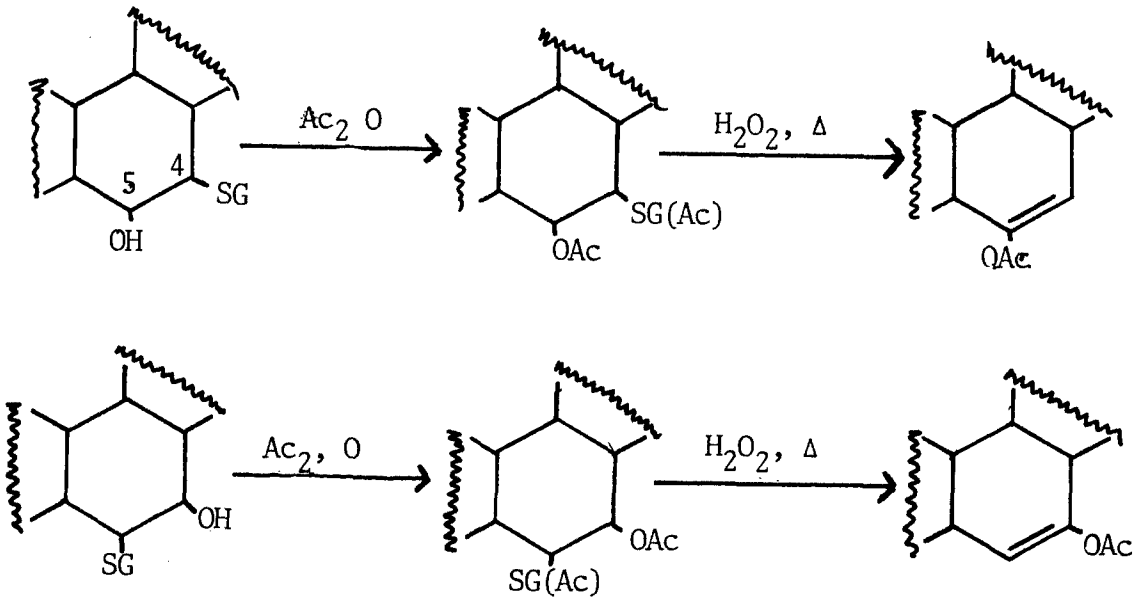


Figure 2. Radioactive profiles of aqueous extracts prepared from 60 min incubations of ^{14}C-naphthalene with lung or liver slices. Conditions are the same as in Figure 1.

benzo(a)pyrene-4,5-oxide. A schematic diagram of the synthetic strategy is shown below:



To prepare 1- and 2-acetoxynaphthalene standards, 3.2 ml acetic anhydride was added dropwise to pyridine solutions of 1 or 2-naphthol (2.4 g in a 25 ml). The reactions were heated at 60 °C for 5 hours. The contents of the reaction vessel were acidified to pH 2 with ice cold 10% HCl and 1- or 2-acetoxynaphthalene and were extracted with equal volumes of chloroform. The chloroform layer was dried over anhydrous sodium sulfate and evaporated to dryness to leave light brown crystals. High pressure liquid chromatography on a C_{18} Radial Pak column (0.5 x 10 cm) in 50% methanol/50% water at 2 ml/min yielded peaks eluting at 2.8, 2.8, 4.5 and 5.4 min for 1- and 2-naphthol and 1- and 2-acetoxynaphthalene, respectively.

1- and 2-acetoxynaphthalene have been separated and identified by gas chromatography/mass spectrometry using the following conditions:

Instrument -- Hewlett Packard 5990A GC/MS (EI)
Column -- 3% OV-1 on 80/100 Chromasorb W HP
Carrier Gas -- Helium, flow rate 40 ml/min
Initial Temperature -- 114°C
Initial Temperature Hold -- 3 min
Program Rate -- 2 °/min
Final Temperature -- 280°C
Injector Temperature -- 260°C

The gas chromatographic and mass spectral characteristics of 1- and 2-acetoxynaphthalene are presented in Table 1. The data in this table indicate that 1- and 2-acetoxynaphthalene can be identified not only on the basis of their retention times but on the relative abundance at 115, 116 and 186.1.

Table 1

Gas Chromatographic and Mass Spectrometric Characteristics of 1- and 2-Acetoxynaphthalene

Compound	Retention Time	m/e	Relative Abundance[a]
1-acetoxynaphthalene	13.6 min	43.0	41.3
		61.9	5.8
		62.9	12.6
		89.0	11.7
		115.0	62.0
		116.0	35.1
		144.1	100.0
		145.1	9.6
		186.1	10.1
2-acetoxynaphthalene	14.6 min	42.1	7.9
		43.0	29.5
		43.9	8.1
		61.9	10.7
		62.9	10.5
		64.9	10.9
		89.0	8.2
		115.0	38.7
		144.1	100.0
		145.1	19.8
		186.1	24.5

[a]Only peaks with a relative abundance of 5% or greater are listed.

Approximately 50 nmoles of each of the putative glutathione conjugates (^{14}C-labeled) were dissolved in 100 µl pyridine, and 100 µl acetic anhydride were added. The reaction mixture was heated at 60°C for 20 min in a reactivial and the excess solvent/reactant was removed under nitrogen. The samples were then dissolved in 20 µl acetone, 2 µl 30% H_2O_2 and 10 µl water. The samples were stirred at room temperature for 48 hours, solvent was removed under nitrogen and each sample was redissolved in 200 µl pyridine. Scintillation counting of 20 µl of this final extract showed that there was little label lost during the acetylation and oxidation steps. Gas chromatography/mass spectrometry of 1-3 µl aliquots of each sample failed to yield peaks corresponding to 1 or 2-acetoxynaphthalene. Thus, further steps to identify the naphthalene glutathione adducts will have to depend upon Raney nickel hydrolysis or proton NMR.

HEPATIC FORMATION, EXPORT AND COVALENT BINDING OF REACTIVE NAPHTHALENE METABOLITES IN EXTRAHEPATIC TISSUES IN VIVO

Studies described in the 1981 annual report indicated that reactive naphthalene metabolites formed in the liver may efflux and cause glutathione depletion and covalent binding in other tissues. If these circulating reactive metabolites are toxicologically important, alterations in hepatic activation of naphthalene would be expected to result in changes in the pulmonary bronchiolar damage by naphthalene. On the other hand, if they are unrelated to pulmonary damage, alteration in pulmonary covalent binding in vivo would not necessarily correspond to changes in the pulmonary damage. Several approaches have been taken in the past contract year to determine what role, if any, the liver plays in the formation of reactive metabolites of naphthalene which become bound covalently and/or cause bronchiolar damage in the lung.

Comparison of Rates of Covalently Bound Naphthalene Metabolites In Vitro with In Vivo Covalent Binding

If metabolites bound in a particular tissue are being formed in situ within that tissue, the levels of covalent binding observed in vivo should correspond reasonably well with the rate at which bound metabolites are formed in vitro. Thus, an experiment was conducted to determine the relative rates of reactive metabolite formation in lung, liver and kidney microsomes. Microsomes were prepared as described previously (Buckpitt and Boyd, 1980) from lung, liver and kidney of male Swiss Webster mice (25-30 g). In a total volume of 2 ml, incubations contained microsomes (4 mg), ^{14}C-naphthalene (0.5 mM, 1064 dpm/nmole), NADPH generating system and $MgCl_2$ (15 µmoles). Control incubations contained no cofactor. After incubation for 15 min at 37°C, the reaction was stopped by the addition of ice cold methanol. Following removal of unbound radioactivity by exhaustive solvent washing, the precipitated microsomal protein pellet was dissolved in 1 N NaOH, an aliquot was taken for protein determination and a further aliquot was added to 5 ml ACS and counted for 20 min in a Beckman 3150T liquid scintillation counter. The data in Table 2 compare covalent binding in vivo with the rate of microsomal, NADPH-dependent covalent binding.

The data showing that in vivo covalent binding levels in the kidney are identical to the liver but that the rate of kidney microsomal activation of naphthalene is only 3.5% that in the lung supports the view that reactive metabolites from naphthalene are sufficiently stable to circulate and become bound covalently in extrahepatic tissues.

Table 2

Comparison of the Relative Rates of Reactive Metabolite Formation by Lung, Liver and Kidney Microsomes In Vitro and the Levels of Covalent Binding In Vivo

Tissue	Covalent Binding (as a percentage of lung binding) In Vivo[a]	In Vitro[b]
Lung	100	100
Liver	133	71.2
Kidney	138	3.5

[a]Data for the in vivo experiments taken from Warren et al. (1982) 4 hours after a 400 mg/kg dose of ^{14}C-naphthalene.

[b]Values for in vitro covalent binding are the means of results from two separate experiments in which 2-3 incubations were run per tissue. Binding in identical incubations containing no NADPH was less than 5% of the value in samples containing cofactor and has been subtracted. Control values for lung microsomal covalent binding were 12.5 (n=2) and 16.4 ± 0.2 ($\bar{x}$ ± S.E., n=3) nmoles/mg protein/15 min.

Effect of Phenobarbital or 3-Methylcholanthrene Pretreatment on the In Vivo and In Vitro Covalent Binding by Reactive Naphthalene Metabolites

Studies outlined in last year's annual report showed that phenobarbital pretreatment followed by 400 mg/kg ^{14}C-naphthalene markedly increased the radiolabel which was bound covalently to tissue macromolecules in lung, liver and kidney compared to vehicle treated controls. Pretreatment with 3-methylcholanthrene increased covalent binding levels in all tissues slightly (not significant at $p < 0.05$) at the 400 mg/kg dose. Neither pretreatment had any effect on covalent binding at the 50, 100 or 200 mg/kg dose of naphthalene, nor did they alter the severity or incidence of lung damage. Data showing that phenobarbital pretreatment has little effect on the pulmonary monooxygenase system (Philpot and Wolf, 1981) yet caused a substantial increase in pulmonary covalent binding further supported the view that reactive naphthalene metabolites circulate from the liver. To confirm that phenobarbital pretreatment did not induce monooxygenases in the lung capable of forming reactive naphthalene metabolites, groups of 25 male Swiss Webster mice (20-25 g) (Charles River) were treated intraperitoneally with vehicle (saline/corn oil), phenobarbital (in 0.1 ml saline/10 g body weight, 10 doses of 50 mg/kg 12 hours apart) of 3-methylcholanthrene (dissolved in corn oil, 2 doses of 80 mg/kg 24 hours apart). The last doses of

3-methylcholanthrene or phenobarbital were given 48 or 24 hours before sacrifice, respectively. Lung (1.75 mg) and liver (4 mg) microsomes, prepared as described, were incubated in the presence of ^{14}C-naphthalene (0.5 mM, 346 dpm/nmole) for 10 min at 37°C. Covalent binding was determined in the precipitated microsomal protein as previously described.

Hepatic microsomal cytochrome P-450 levels, measured by the method of Omura and Sato (1963), were 168 and 149% of vehicle treated controls in phenobarbital and 3-methylcholanthrene treated animals, respectively.

Treatment with either phenobarbital or 3-methylcholanthrene significantly decreased the rate at which lung microsomes catalyzed the covalent binding of naphthalene metabolites (Figure 3). In contrast, liver microsomes from phenobarbital but not 3-methylcholanthrene pretreated animals catalyzed the formation of reactive naphthalene metabolites at a significantly higher rate than control microsomes. Data showing that phenobarbital pretreatment markedly enhances the covalent binding in liver microsomes but actually decreases binding in lung microsomes suggest that, in vivo, the marked increase in binding in the lung may be due to metabolites formed in the liver. This view is consistent with the data showing that 3-methylcholanthrene does not alter the rate of reactive metabolite formation in liver microsomes in vitro and does not significantly affect covalent binding to lung, liver or kidney macromolecules in vivo.

Selective Alteration of Pulmonary Cytochrome P-450 Monooxygenase Activity by p-Xylene Pretreatment

The results of studies outlined in the last annual progress report (Buckpitt, 1981) demonstrated that large doses of p-xylene administered 16 hours prior to sacrifice substantially decreased pulmonary microsomal cytochrome P-450 levels without apparently affecting hepatic cytochrome P-450. In addition, doses of p-xylene (1 g/kg) administered 16 hours prior to administration of 400 mg/kg naphthalene protected against the bronchiolar damage. However, treatment with large doses of p-xylene decreased the covalent binding of naphthalene metabolites in lung, liver and kidney.

These data, showing that the selective effects of p-xylene treatment were not reflected in preferential decreases in pulmonary covalent binding in vivo, suggested that the very large doses of p-xylene could be competitively inhibiting the hepatic metabolism of naphthalene in vivo. Studies were done, therefore, to determine whether lower doses or longer time intervals between p-xylene and naphthalene treatment could be used to destroy pulmonary cytochrome

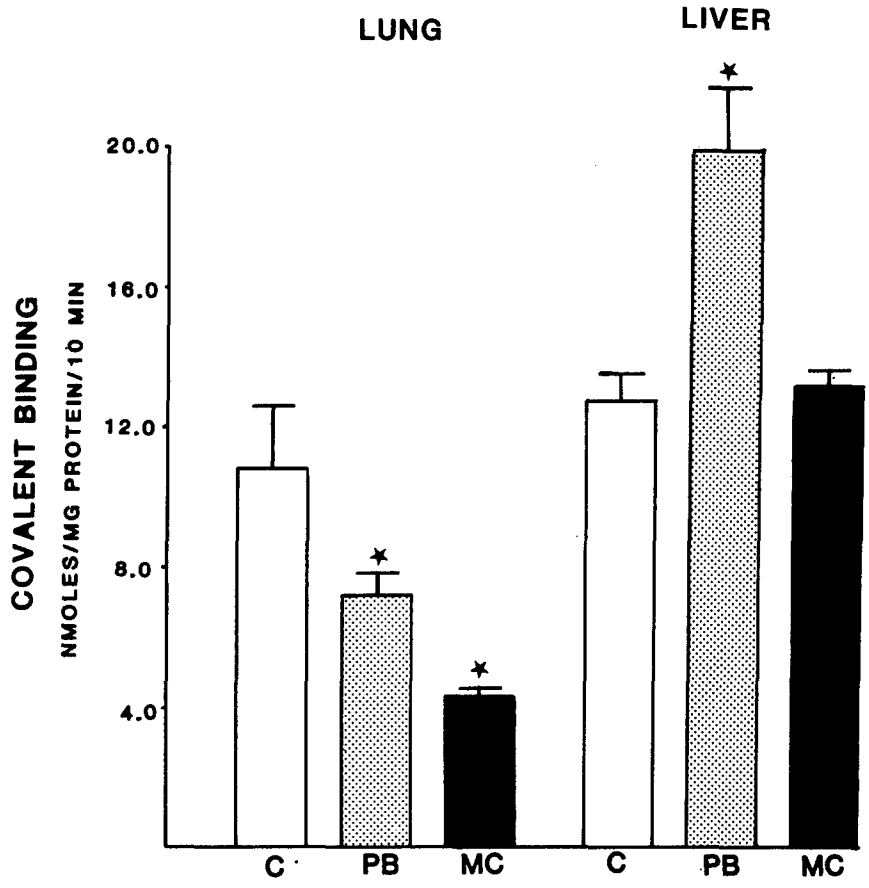


Figure 3. NADPH dependent covalent binding of reactive naphthalene metabolites in lung or liver microsomes from control (C), phenobarbital (PB), or 3-methylcholanthrene (MC) treated mice. Values are the mean ± S.E. for 3 incubations; * indicates a significant difference from controls at $p < 0.05$.

P-450 activity without affecting hepatic metabolism of naphthalene in vivo.

Groups of 8 male Swiss Webster mice (24-33 g) were treated with corn oil or with 125, 250, 500 or 1000 mg/kg p-xylene dissolved in corn oil (0.1 ml/10 g body weight) intraperitoneally. Animals were sacrificed 16 hours after p-xylene administration, and lung and liver microsomes were prepared for determination of cytochrome P-450 dependent metabolism of biphenyl to 4-hydroxybiphenyl. In a total volume of 1 ml, incubations consisted of 1 mg lung or liver microsomes, 1.0 mM biphenyl (added in 10 µl acetone), NADPH regenerating system, $MgCl_2$ (5 µmoles) and were done in 0.1 M sodium phosphate buffer, pH 7.4. After 5 min (liver) or 15 min (lung) of incubation at 37°C, the reaction was quenched with 1.0 ml acetonitrile, protein was removed by centrifugation, and an aliquot was injected onto a 5 µ Radial Pak C_{18} column (0.8 x 10 cm) eluted with 0.02 M KH_2PO_4/acetonitrile (50:50) at 2 ml/min. This procedure is a slight modification of the method of Yamazoe et al. (1981).

The data in Table 3 indicate that doses of 1 g/kg must be administered before a substantial decrease in pulmonary cytochrome P-450 monooxygenase-dependent metabolism of biphenyl is observed. Biphenyl hydroxylase activities in liver microsomes either were unaltered or increased slightly with administration of p-xylene.

Table 3

Dose Response Effect of p-Xylene on Biphenyl 4-Hydroxylase Activity in Mouse Lung and Liver Microsomes

Dose of p-Xylene mg/kg	Biphenyl Hydroxylase[a] Lung	% Control	Liver	% Control
Control	2.34 ± 0.11		10.75 ± 0.10	
125	2.46 ± 0.01	105	11.32[b]	105
250	2.29 ± 0.02	98	10.58[b]	98
500	2.04 ± 0.05	87	11.78 ± 0.24	110
1000	1.61 ± 0.03	69	12.05 ± 0.18	112

[a]Values reported are nmoles/mg protein/15 min (lung) or 5 min (liver) and are the mean ± S.E. for 3 incubations. Standard curves, prepared by adding 4-hydroxybiphenyl to heat inactivated microsomes, were linear over the range of sample values.

[b]Average from 2 incubations.

To determine whether the possible problem of competitive inhibition of naphthalene metabolism in vivo by high doses of p-xylene could be circumvented by increasing the time interval between p-xylene and naphthalene administration, groups of 7 male Swiss Webster mice (Charles River) weighing 21-31 g were treated intraperitoneally with 1 g/kg p-xylene and were sacrificed 24, 36 or 48 hours later. Biphenyl hydroxylase activity was assayed in lung and liver microsomes of animals sacrificed at 24 and 48 hours; NADPH dependent metabolic activation of ^{14}C-naphthalene to covalently bound products was assayed in lung and liver microsomes from animals sacrificed at 24, 36 or 48 hours after p-xylene.

The data in Tables 4 and 5 indicate that p-xylene administration results in a substantial decrease in pulmonary biphenyl hydroxylase both at 24 and 48 hours after administration and that pulmonary microsomal metabolic activation of naphthalene was markedly decreased at 24, 36 and 48 hours. p-Xylene administration increased hepatic microsomal biphenyl hydroxylase slightly but had no effect on the NADPH dependent metabolic activation of naphthalene in liver microsomes.

Table 4

Effect of Varying Time Intervals Between p-Xylene Administration and Sacrifice on Pulmonary and Hepatic Microsomal Biphenyl 4-Hydroxylase

Treatment Group	Biphenyl Hydroxylase[a] Lung	% Control	Liver	% Control
Control	1.72 ± 0.06		11.61 ± 0.66	
24 hr	1.31 ± 0.01[b]	76	15.59 ± 2.21	134
48 hr	1.29 ± 0.01[b]	75	13.10 ± 0.21[b]	113

[a]Values for biphenyl hydroxylase are the mean ± S.E. for 3 separate incubations and are reported as nmoles/mg protein/5 min (liver) or 15 min (lung).

[b]Significantly different from control $p<0.05$ (two tailed student's t test).

Table 5

Effect of Varying Time Intervals Between p-Xylene Administration and Sacrifice on Pulmonary and Hepatic Microsomal Metabolism of Naphthalene to Covalently Bound Metabolites

Treatment Group[b]	Covalent Binding[a] Lung	% Control	Liver	% Control
Control	9.41 ± 0.33		6.77 ± 0.22	
24 hr	5.00 ± 0.17	53.1	6.04 ± 0.23	89.2
36 hr	5.57 ± 0.23	59.2	6.68 ± 0.20	98.7
48 hr	5.22 ± 0.32	55.5	6.75 ± 0.05	99.7

[a]Covalent binding (expressed as nmoles bound/mg protein/10 min) was measured from 2 ml incubations containing: 2.3 mg (lung) or 2.5 mg (liver) microsomal protein, NADPH generating system and 0.5 mM ^{14}C-naphthalene (1000 dpm/nmole). Controls were run in the absence of NADPH to correct for a small amount of binding (less than 2% of that in cofactor samples).

[b]Doses of 1 g/kg p-xylene were administered in corn oil at the specified time before sacrifice.

Thus, studies were done to determine whether administration of p-xylene (1 g/kg) 48 hours prior to the administration of naphthalene would block the bronchiolar damage and/or would preferentially alter glutathione depletion or covalent binding of reactive naphthalene metabolites in the lung.

Groups of 5 mice each (21-31 g) (Charles River) were treated ip with either corn oil or p-xylene (1 g/kg) followed 48 hours later by corn oil or naphthalene (300 mg/kg). Lungs, removed 24 hours later, were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 5 µm and stained with hematoxylin and eosin. Tissue damage was assessed by light microscopy. Bronchiolar damage was not observed in corn oil or p-xylene treated animals. Typical necrosis and exfoliation of bronchiolar epithelial cells was noted in animals treated with corn oil plus naphthalene. Pretreatment with p-xylene failed to alter either the severity or incidence of this lesion.

An additional 4 groups of 5 mice each were treated ip with corn oil or p-xylene (1 g/kg) followed 48 hours later by corn oil, or ^{14}C-naphthalene (300 mg/kg, 134 dpm/nmole). All animals were sacrificed 4 hours after the last injection for determination of non-protein sulfhydryl (glutathione) and covalent binding. Non-protein

sulfhydryl concentrations were determined by the method of Ellman (1959); covalent binding of radiolabel was assessed in the precipitated macromolecular fraction after exhaustive solvent washing (Warren et al., 1982).

The data in Figure 4 show that p-xylene treatment caused a slight decrease in glutathione levels when compared to corn oil controls. Prior treatment with p-xylene failed to block the glutathione depletion by 300 mg/kg naphthalene and caused a slight, but not significant, decrease in covalent binding in lung and kidney. Thus, at a dose and time after p-xylene administration which causes a 45% decrease in the metabolic activation of naphthalene by lung microsomes in vitro, there was little change in the formation of reactive metabolites from naphthalene (assessed by glutathione depletion and covalent binding) in the lung in vivo. The results of these studies are again consistent with the view that reactive metabolites of naphthalene which are bound covalently in the lung can be formed by the liver.

Selective Glutathione Depletion in Liver and Kidney - Effect of Pretreatment with Buthionine Sulfoximine on Naphthalene-Induced Bronchiolar Damage and on the Covalent Binding and Glutathione Depletion by Reactive Naphthalene Metabolites

Studies by Meister et al. (1980) and Griffith and Meister (1979) have shown that subcutaneous administration of buthionine sulfoximine (4 mmoles/kg) results in selective depletion of glutathione in liver and kidney but not lung. If reactive metabolites from naphthalene circulate from the liver, treatment with buthionine sulfoximine would be expected to increase the covalent binding of naphthalene metabolites in lung as well as liver and kidney. Likewise, if metabolites circulating from the liver are toxicologically important, buthionine sulfoximine pretreatment should increase the severity of the lung lesion by naphthalene.

Buthionine sulfoximine (melting point 211-212°C) was prepared and generously supplied by Dr. Ronald Shank and his coworkers, University of California, Irvine. The synthesis, purification and recrystallization were done according to the procedures of Griffith and Meister (1979). To determine whether selective depletion of glutathione would alter the pulmonary covalent binding or bronchiolar damage by naphthalene, groups of 5 mice each were treated subcutaneously with either saline or buthionine sulfoximine (880 mg/kg, dissolved in saline, 0.1 ml/kg body weight). Two hours later a saline and buthionine sulfoximine treated group were sacrificed for glutathione determination. An additional six groups treated with saline or buthionine sulfoximine were given corn oil, naphthalene 100 mg/kg or naphthalene 200 mg/kg ip and were sacrificed 24 hours later.

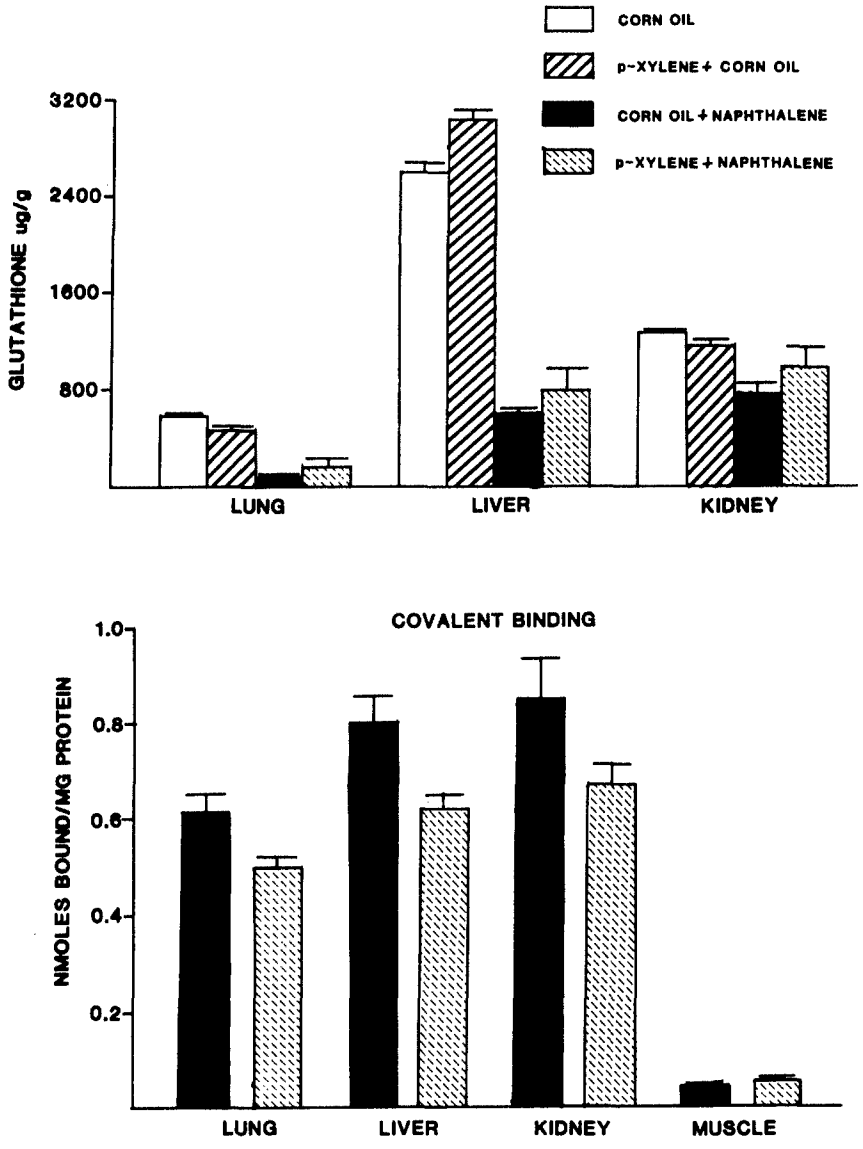


Figure 4. Effects of p-xylene treatment on naphthalene induced depletion of tissue glutathione and on the covalent binding of naphthalene metabolites. Values are the mean ± S.E. for 5 animals.

Lung, liver and kidney were removed, fixed in buffered formalin and processed for light microscopic examination. The data presented

in Table 6 indicate that lungs from mice treated with buthionine sulfoximine plus corn oil were indistinguishable from animals treated with corn oil alone.

Table 6

Effect of Buthionine Sulfoximine Pretreatment on Naphthalene Induced Pulmonary Bronchiolar Damage

Pretreatment	Dose of Naphthalene mg/kg	% Mortality	Bronchiolar Necrosis
Saline	0	0	No
	100	0	No
	200	0	Yes
Buthionine sulfoximine	0	0	No
	100	0	Yes
	200	80	No[a]

[a]Four of the five mice were dead within 6 hours of the dose of naphthalene. Bronchiolar lesions were not present in the one animal surviving 24 hours.

Consistent with previous studies, bronchiolar lesions were not observed in mice treated with saline plus 100 mg/kg naphthalene; marked swelling and some exfoliation of terminal bronchiolar epithelial cells were observed in lungs of mice treated with saline plus 200 mg/kg naphthalene. In contrast, the terminal bronchiolar airways of mice treated with buthionine sulfoximine followed by 100 mg/kg naphthalene were moderately damaged with epithelial cell swelling in most terminal bronchioles. Exfoliation also was observed in some terminal airways. Eighty percent of the animals treated with buthionine sulfoximine plus 200 mg/kg naphthalene were dead within 6 hours of hydrocarbon administration; no lung lesions were observed in the one animal surviving 24 hours suggesting that this animal was not treated with naphthalene. Liver and kidney necrosis were not observed in any of the treatment groups.

Two additional groups of 5 animals each were treated subcutaneously with either saline or buthionine sulfoximine (880 mg/kg) followed by ^{14}C-naphthalene (200 mg/kg, 313 dpm/nmole). Animals were sacrificed 4 hours later for determination of non-protein sulfhydryl (glutathione) (Ellman, 1959) and covalent binding of radiolabel to tissue macromolecules (Warren et al., 1982). The data in Figure 5 show that two hours after buthionine sulfoximine treatment, glutathione levels were decreased significantly in the

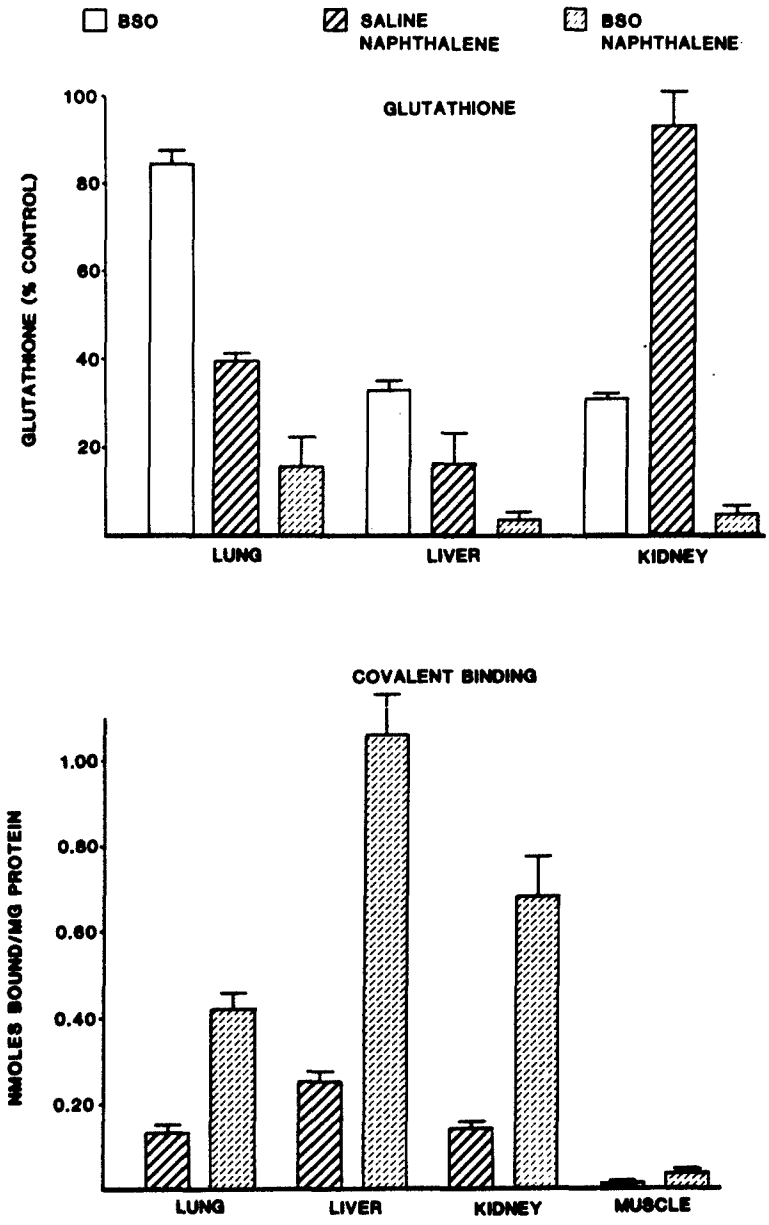


Figure 5. Effect of buthionine sulfoximine treatment on glutathione levels and on the covalent binding of reactive naphthalene metabolites. Values are the mean ± S.E. for 5 animals. Control glutathione levels were 694 ± 45 µg/g, 2621 ± 108 µg/g and 1445 ± 33 µg/g for lung, liver and kidney, respectively ($\bar{x}$ ± S.E.).

liver and kidney (32 and 30% of saline treated controls, respectively) but not in lung (85% of control). Treatment with saline plus 200 mg/kg naphthalene resulted in a decrease in glutathione levels in lung and liver but not kidney. Pretreatment with buthionine sulfoximine followed by naphthalene further depressed glutathione levels in lung and liver compared to the saline plus naphthalene treated groups. Likewise, glutathione levels in the kidney were markedly lower in the buthionine sulfoximine plus naphthalene treated group than they were in the groups treated with buthionine sulfoximine plus corn oil or saline plus naphthalene.

Covalent binding in lung, liver and kidney, which is relatively low in animals treated with saline plus ^{14}C-naphthalene (200 mg/kg) is increased substantially in all three tissues by buthionine sulfoximine treatment (Figure 5). The fact that buthionine sulfoximine increases binding in lung as well as liver and kidney suggests that reactive metabolites of naphthalene which are bound in the lung may originate in the liver. A further possibility which must be considered in evaluating the data is that buthionine sulfoximine treatment blocks a major detoxication pathway for naphthalene in the liver. Thus, the liver may clear less of an ip dose of the hydrocarbon thereby resulting in higher levels of naphthalene available for metabolic activation in the lung.

Inhalation Studies

A further approach to determining the relative roles of hepatic and pulmonary monooxygenase enzymes in the formation of reactive metabolites from naphthalene has been to study the differences in lung damage and in the formation of electrophilic naphthalene metabolites after administration by intraperitoneal and inhalation routes. In addition, inhalation is one of the major routes of human exposure, and it was important to determine whether the characteristic bronchiolar necrosis observed after ip administration of the compound would result from inhalation exposure.

Preliminary static exposures indicated that 3 hour exposures to concentrations of naphthalene as low as 8 ppm (44 mg/m^3) caused substantial damage to the bronchiolar epithelium (Buckpitt, 1981). Because the exposures were conducted in a static chamber, they were not ideal from several standpoints. It is likely that CO_2 concentrations at the end of the exposure were sufficiently high to affect breathing rate and that naphthalene remaining on the animals' fur could be preened and ingested orally.

A small volume, high containment nose-only manifold capable of exposing 8 mice simultaneously (Figure 6) was designed and constructed with the aid of Drs. Robert Phalen (Director, Air

Pollution Health Effects Laboratory) and Ronald Rasmussen. From the point of generation to the gas washing bottles, all surfaces were glass, brass or copper to minimize the absorption of naphthalene into plastic or rubber. Chamber concentrations were controlled by relative flow rates through the generation and dilution air rotometers and by the amount of heat applied to the generation flask. A slight negative pressure was maintained in the chamber (0.0 to 0.2 inches of water, monitored with a magnahelic). The outflow from the chamber was passed through 2 gas washing bottles containing hexane. Chamber flow rates were 1 ℓ/min.

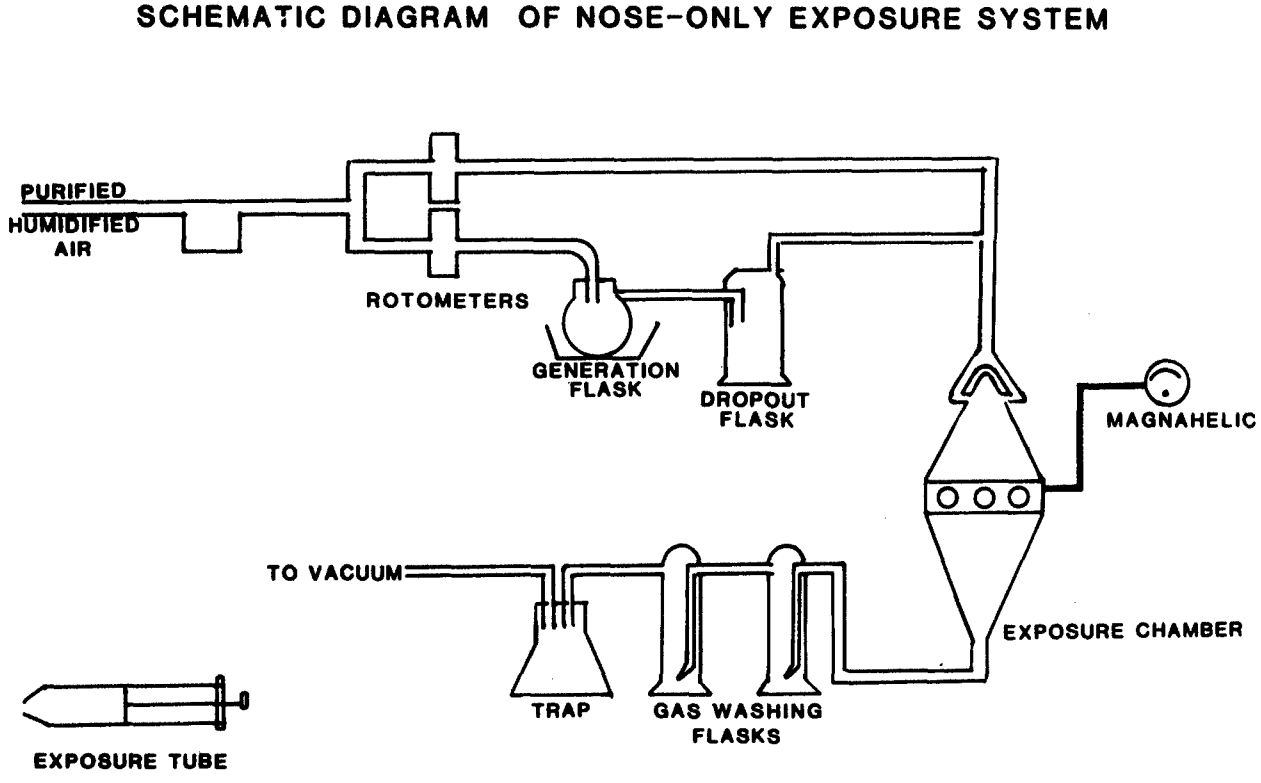


Figure 6. Schematic diagram of nose only inhalation manifold.

Chamber concentrations were monitored by removing a 10 ml air sample (through a GC septum) with a gas tight syringe and bubbling the sample into 1 ml spectral grade trimethylpentane (this procedure has been shown to result in the recovery of virtually 100% of the naphthalene). An aliquot of the trimethylpentane was then subjected to high pressure liquid chromatography on a 10 µ radial pak silica column (5 x 100 mm) with trimethylpentane as the mobile phase at 1.5 ml/min. Naphthalene eluted in 2 min (the column void volume is 1.4 ml). Standard curves prepared with authentic naphthalene were linear over the range tested (4.6 to 46 ng injected).

Dose Response Histopathology

Four groups of 5 mice each were exposed for 1 hour to concentrations of 52.4, 95.8, 204.3 and 379.9 µg/ℓ naphthalene, and the animals were sacrificed 24 hours later. Lungs were removed, fixed in buffered formalin, embedded in paraffin, sectioned at 5 µm and stained with hematoxylin and eosin. Light microscopic examination of the lungs showed only slight damage (swelling, some sloughing of cells of the larger airways of animals exposed at the highest-dose). Cells of the terminal bronchiolar airways were unaffected by even the highest exposure concentration (380 µg/ℓ). Thus, the dose response inhalation exposure studies were repeated, this time extending the length of exposure to 4 hours. In addition, groups of mice were pretreated either with piperonyl butoxide (600 µl/kg) or with diethyl maleate (600 mg/kg) 30 min prior to the start of the exposures. Table 7 summarizes the data from this experiment.

In comparison to exposures conducted under static conditions, bronchiolar damage after nose only exposure was observed only at considerably higher doses. As discussed previously, this difference may be due to oral ingestion during and after the exposure and higher breathing rates in statically exposed animals. Similar to the results obtained after ip administration, pretreatment with piperonyl butoxide decreased and diethylmaleate increased the pulmonary damage by naphthalene administered by inhalation.

To determine whether glutathione depletion and/or covalent binding of reactive metabolites from naphthalene occurs after inhalation exposure, groups of 4 male Swiss Webster mice (Charles River) were treated either with corn oil (0.1 ml/10 g body weight) or diethylmaleate (600 µl/kg dissolved in corn oil) 30 minutes prior to the start of inhalation of ^{14}C-naphthalene. A group of 5 animals treated with corn oil was exposed to clean air only and served as glutathione controls. Animals were exposed on consecutive days to concentrations of ^{14}C-naphthalene of 540, 370 or 130 µg/ℓ (99 dpm/nmole) for 4 hours. All exposures were started between 11:30 and noon to minimize the effects of diurnal variation on tissue

Table 7

Pulmonary Damage by Naphthalene Administered by Inhalation

Group	Exposure Concentration[a] µg/ℓ ± S.E.	Pretreatment	Lung Damage[b] None	Mild	Moderate	Severe
I	376 ± 10	None		2/5	3/5	
II	384 ± 7	Piperonyl Butoxide	1/5	4/5		
III	175 ± 9	None	3/5	2/5		
IV	93 ± 4	None	3/5	2/5		
V	101 ± 2	Diethylmaleate				5/5

[a]Concentrations are the mean ± S.E. for samples taken every 15 min over the four hour sampling period.

[b]Mild damage corresponds to swelling of some cells either in major or terminal airways; moderate damage denotes lungs in which some cells in either major or terminal airways were sloughed into the airway lumen; and severe damage corresponds to lungs in which the majority of cells in the terminal airway were sloughed into the bronchiolar lumen.

glutathione levels. Animals were sacrificed two hours postexposure for determination of tissue glutathione levels (animals treated with corn oil) and covalent binding (all animals except air exposed).

The data in Table 8 indicate that even at the highest concentration of naphthalene tested, there was only slight depletion of pulmonary glutathione. Moreover, quantitatively similar amounts of depletion were observed in the 130 and 540 µg/ℓ exposure groups. It is possible that, even at the 130 µg/ℓ dose of naphthalene, all of the glutathione in the lung cells capable of metabolically activating this hydrocarbon was depleted.

These results provide further evidence that the substantial depletion of pulmonary glutathione occurring after ip administration of high doses of naphthalene is caused by reactive metabolites effluxing from the liver. Moderate glutathione depletion was observed in liver, and again this effect was not dose-related. Likewise, renal glutathione was slightly depressed at all doses of naphthalene.

The levels of covalent binding were highest in lung with much lower levels in liver, kidney and muscle (Table 9). Similar to the

Table 8

Effect of Naphthalene Administration by Inhalation on Tissue Reduced Sulfhydryl Levels

Dose µg/ℓ[b]	Glutathione µg/g tissue[a] Lung	Liver	Kidney
540	379.1 ± 3.9 (75)	1402.4 ± 45.6 (62)	1186.1 ± 22.7 (88)
370	336.9 ± 56.7 (67)	1446.0 ± 41.2 (64)	1046.1 ± 106.2 (78)
130	380.1 ± 27.0 (75)	1419.2 ± 67.2 (63)	1046.2 ± 86.7 (78)
Clean Air	502.8 ± 19.0	2253.7 ± 78.3	1339.8 ± 22.7

[a]Values are the mean ± S.E. for 4 animals. Numbers in parentheses indicate values as a percentage of air exposed controls.

[b]Doses are the mean of samples taken every 15 min. Standard errors were less than 7% of the mean.

Table 9

Covalent Binding of Radiolabel from ^{14}C-Naphthalene After Inhalation: Dose Response and Effect of Diethylmaleate Pretreatment

Dose µg/ℓ	DEM	Covalent Binding nmoles/mg protein Lung	Liver	Kidney	Muscle
540	+	0.35 ± 0.03	0.07 ± 0.01	0.09 ± 0.02	0.01
	-	0.34 ± 0.03	0.02 ± 0.01	0.06 ± 0.01	0.01
370	+	0.28 ± 0.04	0.04 ± 0.01	0.05 ± 0.02	ND
	-	0.32 ± 0.05	0.03 ± 0.01	0.06 ± 0.02	ND
130	+	0.17 ± 0.03	0.01 ± 0.00	0.02 ± 0.00	ND
	-	0.30 ± 0.03	0.01 ± 0.00	0.03 ± 0.00	ND

[a]Covalent binding is expressed as nmoles bound/mg protein. Values are the mean ± S.E. for 4 animals. ND = not detected.

results with glutathione depletion, the dose-response curves were very flat. Tripling the dose of inhaled naphthalene from 130 to 540 µg/ℓ increased covalent binding in the lung from 0.30 to only 0.34 nmoles/mg protein. Again, it is possible that, even at the

lowest exposure concentration, the amount of inhaled naphthalene was sufficient to saturate the pulmonary P-450 enzymes. Another possibility in explaining the very flat dose-response curves may be that the amount of naphthalene actually absorbed by the lung may remain relatively constant over the dose range tested. Similar flat dose-response curves for the covalent binding of reactive metabolites to liver macromolecules have been obtained with vinyl chloride, trichlorethylene and carbon tetrachloride by Bolt and Filser (1977).

Pretreatment with the glutathione depletor, diethylmaleate, caused a sharp decrease in covalent binding at low exposure concentrations and had no effect at the higher exposure concentration. These results are surprising in light of the observed increase in bronchiolar damage in diethylmaleate pretreated compared to vehicle pretreated animals and will have to be confirmed by further experiments. Glutathione levels rebound sharply after pretreatment with agents that deplete glutathione (Buckpitt et al., 1982). Thus, it is possible that 2 to 3 hours after treatment with diethylmaleate, pulmonary glutathione levels are higher than in corn oil treated animals thereby resulting in lower levels of covalently bound metabolites in groups exposed to low concentrations of naphthalene.

POSSIBLE COVALENT BINDING OF NAPHTHALENE METABOLITES TO DNA

The covalent binding of electrophiles to DNA is generally thought to play a critical role in chemical carcinogenesis (Lutz, 1979). Since there is substantial evidence suggesting that naphthalene is metabolized in vivo to epoxide, diepoxide and diol epoxide derivatives and because our data indicate that considerable cytochrome P-450 dependent covalent binding of naphthalene metabolites occurs to cellular nucleophiles in vivo and in vitro, it is possible that a fraction of these metabolites form adducts with DNA.

DNA Binding of Reactive Naphthalene Metabolites In Vitro

To determine whether reactive metabolites of naphthalene formed in liver microsomal incubations are capable of binding covalently to exogenously added DNA, liver microsomes were prepared from male Swiss Webster mice treated for 7 days with phenobarbital (0.1% in the drinking water). In a total volume of 6 ml, incubations contained ^{14}C-naphthalene (0.5 mM, 5 mCi/mmole), 4 mg liver DNA, microsomes (12 mg protein) and NADPH regenerating system. Control incubations were done in the absence of NADPH. Following incubation at 37°C for 30 min, an equal volume of phenol reagent (75 ml m-cresol, 0.5 g 8-hydroxyquinoline, 600 g phenol in 55 ml water) was added, and DNA was extracted by a procedure similar to that of Lai et al. (1979).

After shaking for 30 min at room temperature, samples were centrifuged for 10 min at 13,000 x g. The upper layer was removed, an equal volume of ice cold absolute ethanol was added and the samples were allowed to remain in the freezer overnight. The DNA was pelleted by centrifugation at 1000 x g for 20 min, and the ethanol supernatant was removed. The remaining ethanol was evaporated under a gentle stream of nitrogen. Dried DNA was dissolved in SSC (1.5 mM sodium citrate, 15 mM sodium chloride pH 7.0) and incubated with preheated ribonuclease (50 µg/ml) and 10 µmoles $MgCl_2$ for 30 min at 37°C. Following this, DNA was incubated for 60 min at 37°C with Pronase (100 µg/ml). At the end of the incubation, an equal volume of phenol reagent was added, and DNA was extracted by shaking at room temperature for 30 min. After centrifuging the samples at 13,000 x g for 30 min, the upper phenol layer was removed, and DNA was washed five times with ice cold ethanol; radioactivity in 1 ml of the final ethanol wash was near background. The ethanol supernatant was removed, and the last traces of solvent were evaporated under a gentle stream of nitrogen. The DNA was dissolved in SSC, an aliquot was taken for determination of absorbance at 260 nm and a further aliquot was added to a scintillation vial. DNA in the scintillation vial was incubated in 500 µg DNase for 30 min at 37°C. Fifteen ml ACS scintillation fluid was added, the vials were cleaned and samples were counted for 100 min in a Beckman 3150T liquid scintillation counter. All samples were counted to 3% error or better. Efficiency was determined by spiking each sample with 15 µl ^{14}C-toluene standard (14,962 dpm). DNA content was calculated by the absorbance value at 260 nm compared to standards prepared from isolated rat liver DNA. The data in Table 10 show that NADPH-dependent metabolism of naphthalene resulted in derivatives which appear to bind covalently to DNA. These data must be interpreted cautiously since the presence of a small amount of protein in the final DNA extract could account for the radioactivity in the samples with cofactor.

This experiment was repeated with the exception that liver microsomes were prepared from control, phenobarbital (50 mg/kg BID x 5 days), or 3-methylcholanthrene (20 mg/kg x 2 days) pretreated mice. Incubations contained 5 mg mouse liver DNA, 2 mg/ml microsomal protein, 0.5 mM ^{14}C-naphthalene (5 mCi/mmole) and NADPH regenerating system in a total volume of 7 ml. Two incubations from each group of microsomes were done without NADPH as controls. DNA was isolated as described in the previous experiment. Dried DNA was dissolved in a total volume of 2 ml 0.1 SSC, and a 200 µl aliquot was removed for Lowry protein determination. The remainder was incubated with DNase, 15ml ACS scintillation fluid was added and samples were counted for 100 minutes. Average counting efficiency determined by internally standardizing each sample was 93.3%. The data in Table 11 show the dpm, A_{260} to A_{280} ratio and Lowry protein value for each sample. Absorbance values for the Lowry protein determination ranged from

Table 10

Covalent Binding of Radioactivity from ^{14}C-Naphthalene to DNA In Vitro[a]

Sample	NADPH	cpm	A_{260}	Covalent Binding pmoles/mg DNA
1	+	212	.385	3.4
2	+	335	.304	7.4
3	-	45	.290	0.3
4	-	45	.573	0.1

[a]Incubations contained in a total volume of 6 ml:phenobarbital induced liver microsomes (2 mg/ml), DNA (liver) 4 mg and ^{14}C-naphthalene. Reagent blank was 34 cpm. Counting efficiency averaged 86.6%.

Table 11

Comparison of Covalent Binding of Radioactivity from ^{14}C-Naphthalene to DNA in Microsomal Incubations from Control Phenobarbital 3-Methylcholanthrene Treated Animals

Sample	Pretreatment	NADPH	DPM	$A_{260/280}$	Total Protein µg/2 ml
1	Control	+	54	1.79	2.1
2		+	67	1.77	6.1
3	Control	-	12	1.75	3.9
4		-	7	1.76	1.7
5	Phenobarbital	+	35	1.79	-
6		+	28	1.72	1.3
7		+	89	1.78	1.7
8		-	4	1.69	17.0
9		-	1	1.70	0.5
10	3-Methyl	+	60	1.79	18.2
11	Cholanthrene	+	44	1.80	2.0
12		+	44	1.78	0.5
13		-	22	1.72	2.0
14		-	4	1.72	-

0.013 to 0.062. Absorbance in this range cannot be determined with utmost confidence. Since the $A_{260/280}$ ratio of pure DNA is >1.90, the data in Table 11 indicate the presence of small amounts of protein. While it is possible that the source of this protein is the ribonuclease or pronase used to digest ribonucleic acids or protein earlier in the isolation procedure, it is not possible to exclude microsomal protein as the source of contamination. Because binding of nucleophilic sites on protein is very high (Table 2) it is not possible to unequivocally attribute the radioactivity isolated with DNA as being bound covalently to DNA bases.

A further experiment, done in collaboration with Dr. Ronald Shank and a senior graduate student in his laboratory, Eric Hunt, has been conducted to determine whether mouse liver DNA, isolated after naphthalene treatment, contained arylated guanine which could be detected by the HPLC-fluorescence procedures developed in Dr. Shank's laboratory. One hundred and thirty mice were fed 0.1% phenobarbital in the drinking water for 6 days. This was replaced with fresh water 24 hours before administering 400 mg/kg naphthalene (80 animals) or corn oil (50 animals) ip. Animals were sacrificed 4 hours later and DNA was isolated by the method of Swann and Magee (1968). Neutral-thermal and mild acid DNA hydrolysates were prepared from control and naphthalene-treated DNA and were chromatographed on a Whatman SCX analytical column in either water (4 min) followed by a step gradient to 0.1 M ammonium phosphate pH 2.0 or in 0.07 M ammonium phosphate, pH 2.0 in 5% methanol. Fluorescence of the eluate was monitored at an excitation wavelength of 287 nm excitation and a 343 nm cut-off filter. These chromatographic/monitoring conditions have been found to be optimal for alkylated guanines from DNA after treatment with methylating or ethylating carcinogens. No fluorescent peaks were observed in liver DNA from mice treated with naphthalene that were not present in controls. Thus, either naphthalene metabolites are not bound to DNA or the conditions used for isolation and analysis were inappropriate.

SUMMARY AND CONCLUSIONS

Prior studies have shown a correlation between the extent of covalent binding of reactive naphthalene metabolites with the severity of pulmonary bronchiolar necrosis induced by this hydrocarbon in mice (Warren et al., 1982). While the levels of covalent binding in the lung correlate with the bronchiolar damage, the tissue selectivity for damage is not reflected in the preferential arylation of pulmonary macromolecules. At all doses and times after administration of ^{14}C-naphthalene, covalent binding is higher in non-target than in target tissues. Moreover, all of the pretreatments studied to date have failed to alter the tissue selectivity for covalent binding. There are several possible

explanations for this lack of tissue specificity for covalent binding which are still consistent with a role of reactive metabolites in initiating those events that eventually lead to necrosis of bronchiolar epithelial cells. Due to the apparently high degree of cellular localization of pulmonary cytochrome P-450, the formation of reactive naphthalene metabolites may occur in a relatively small group of lung cells. Thus, if covalent binding could be expressed on the basis of nmoles bound/cell, it is possible that binding would be considerably higher in lung than in liver. Indeed, comparisons of the rates of covalent binding in lung, liver and kidney microsomes showed the rate of reactive metabolite formation to be substantially higher in lung microsomes than in liver or kidney microsomes (Table 2). Moreover, expressing these data on a per nmole cytochrome P-450 basis would indicate that the rate of reactive metabolite formation by pulmonary microsomal P-450 is 10 to 20 times higher than liver. Since these incubations were done at 0.5 mM naphthalene, a concentration likely to be at or near saturating, it is probable that kinetics of reactive naphthalene metabolite formation in the crude microsomal enzymes differ substantially.

Another possibility in explaining the lack of tissue specificity for covalent binding is that metabolism of naphthalene may result in the formation of a number of different reactive metabolites including the 1,2-oxide, 1,2:3,4 dioxide and the 1,2-dihydrodiol-3,4-epoxide (Horning et al., 1980). All of these metabolites may be capable of binding covalently to macromolecules but they may differ in their ability to interact with macromolecules critical to the survival of the cell. Since the nature of reactive metabolites produced in different tissues could determine the tissue specificity for damage, preliminary studies reported here have begun to examine differences in target vs non-target tissue formation of reactive naphthalene metabolites in vitro. The approach which has been used in these studies is to add glutathione and the glutathione transferases to form water soluble thioether adducts with the reactive metabolites that are generated microsomally. These metabolites can then be separated and quantitated by HPLC. Preliminary comparisons of glutathione adducts formed in liver vs lung slice incubations indicate that not only is the total rate of formation of thioether metabolites in lung slices higher than in liver slices but that extracts from lung slice incubations contain relatively large amounts of a metabolite eluting at 21.2 min which is present in much smaller amounts in extracts from liver slices. Current efforts are aimed at identifying the various thioether metabolites formed in these incubations as this will yield structural information about the precursor reactive metabolites.

Since the liver contains a major fraction of cytochrome P-450 monooxygenase in the mammalian organism, studies dealing with the

relationship of reactive metabolite formation to cytotoxicity or carcinogenesis in extrahepatic tissues must consider the liver as a source of reactive and/or toxic metabolites. Studies with monocrotalin, a pyrolizidine alkaloid which causes pulmonary capillary endothelial damage in rats (Huxtable, 1979) and 3,3-dimethyl-1-phenyltriazene, a central nervous system carcinogen (Bartsch et al., 1977) have shown that metabolism of these compounds in the liver results in the formation of unstable metabolites which are capable of effluxing from the liver and which are critical to the pulmonary or central nervous system toxicity, respectively.

In addition, Smith and his coworkers (1980a,b) have shown that relatively stable aromatic hydrocarbon epoxides like benzo(a)pyrene-4,5-oxide are capable of arylating pulmonary macromolecules when used as a substrate in the isolated perfused lung system. The findings that naphthalene-1,2-oxide has a half life in aqueous media of 4 min (Jerina and Daly) and that glutathione is nearly totally depleted in mouse lung after high doses of naphthalene (Warren et al., 1982) suggested that metabolism in the liver may play an important role in extrahepatic covalent binding by reactive naphthalene metabolites. This view has been supported by a number of additional studies.

Comparison of the levels of covalent binding in vivo with the rates of reactive metabolite formation in vitro indicates that in vivo binding is very high in kidney yet renal microsomal metabolism of naphthalene to reactive metabolites is quite low. Furthermore, pulmonary, hepatic and renal covalent binding are markedly increased in phenobarbital pretreated mice compared to controls after a 400 mg/kg dose of naphthalene (Buckpitt, 1981). However, in vitro, phenobarbital pretreatment significantly decreased the rates of reactive metabolite formation in lung microsomes but increased hepatic microsomal reactive metabolite generation. Thus, the observed increase in pulmonary covalent binding in vivo in phenobarbital pretreated mice appears to result from increased rates of reactive metabolite formation by hepatic monooxygenase. The data obtained in 3-methylcholanthrene pretreated mice support this view. Rates of covalent binding catalyzed by lung microsomes of 3-methylcholanthrene pretreated mice were significantly lower than in vehicle treated animals, but hepatic microsomal activation of naphthalene was similar in control and 3-methylcholanthrene animals. In vivo, covalent binding in 3-methylcholanthrene treated mice was not significantly different from mice treated with vehicle.

The view that reactive metabolites from naphthalene can efflux from the liver and that these metabolites may play a significant role in pulmonary bronchiolar damage was supported by data showing that p-xylene treatment depressed the pulmonary microsomal metabolic

activation of naphthalene and the metabolism of biphenyl significantly but had only slight effects on pulmonary covalent binding in vivo and no effect on the severity of naphthalene-induced bronchiolar damage.

Selective depletion of hepatic glutathione by buthionine sulfoximine treatment resulted in substantial increases in covalent binding of reactive metabolites of naphthalene in lung as well as liver and kidney and in an increase in the bronchiolar damage. Again these results are consistent with hepatic formation of metabolites which become covalently bound to tissue macromolecules in the lung and which may play a causative role in the bronchiolar damage. Depletion of hepatic glutathione blocks a quantitatively important metabolic pathway for naphthalene in the liver and, thus, it is possible that the observed increase in bronchiolar necrosis and covalent binding in buthionine sulfoximine pretreated animals were simply due to a shift in the total amount of parent hydrocarbon reaching the lungs. During the upcoming studies on the disposition of naphthalene it will be important to exclude this possibility.

Bronchiolar necrosis and pulmonary covalent binding of reactive naphthalene metabolites was demonstrated after inhalation exposures to naphthalene. The incidence and severity of bronchiolar damage increased only slightly with a quadrupling of the dose; however, pulmonary damage was increased substantially by depletion of glutathione (diethylmaleate pretreatment) and was decreased slightly by inhibition of monooxygenases (piperonyl butoxide pretreatment). In contrast to the data obtained when naphthalene is administered by intraperitoneal injection, dose response curves for covalent binding and glutathione depletion in the lung were flat. It is possible that, over the dose range tested, the quantity of naphthalene to which airway cells are being exposed is not increasing in proportion to the increase in dose. The observation that diethyl maleate treatment increased the severity of pulmonary damage, yet decreased covalent binding at the low exposure concentrations, is surprising and will require additional study.

Finally, evidence suggesting that reactive metabolites of naphthalene are bound covalently to DNA has been obtained in in vitro studies. These results will require further, more in depth, study to insure that the observed binding was not due to protein contamination of the DNA. It will be important, as well, to assess the possibility of binding of metabolites of naphthalene to DNA in vivo.

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